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14. ABSTRACT
BORIS (CTCFL) is a pa-alogue of the gene encoding CTCF, a multifunctional DNA binding protein that utilizes different sets of zinc fingers to mediate distinct gene regulatory functions, including those involved in cell growth regulation. Unlike CTCF, the expression of BORIS is normally restricted to specific cells in testes (the only cells where CTCF is not expressed), where it may play a role in reprogramming the methylation pattern of male germ line DNA. To define the possible consequences of aberrant BORIS expression in human breast cancers, we have used a well-characterized human mammary epithelial cell (HMEC) culture model. Our results indicate that in most breast cancer cells, endogenous BORIS is unlikely to be expressed at sufficient levels to interfere with CTCF functions, and that BORIS expression alone is not an efficient immortalizing factor. However, under certain conditions BORIS may cooperate with other changes (e.g. p53 inactivation) to destabilize the genomes of the cells in which it is aberrantly expressed. BORIS expression may cause genornic instability through aberrant affects on centrosome duplication during the cell cycle, and through effects on the regulation of several key early growth response genes.

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INTRODUCTION

Recently, a new gene was mapped to the 20q13.2 region that is commonly amplified in cancers of the breast and other tissues. This gene, BORIS (Brother of the Regulator of Imprinted Sites), is a paralogue of the gene encoding CTCF, a multifunctional DNA binding protein that utilizes different sets of zinc fingers to mediate distinct functions in regulation of gene expression (reviewed in (1)). These functions include context-dependent promoter repression or activation, creation of modular hormone-responsive gene silencers, and formation of enhancer blocking elements (insulators). Several lines of evidence suggest that ubiquitously expressed CTCF is a critical determinant of cell growth regulation.

Unlike CTCF, which is expressed ubiquitously, the expression of BORIS is normally restricted to specific cells in testes (the only cells where CTCF is not expressed), where it may play a role in reprogramming the methylation pattern of male germ line DNA (2). BORIS encodes an 11-zinc finger domain functionally equivalent to that in CTCF, while being completely divergent at the amino- and carboxy-termini – domains that have recently been shown to be critical for CTCF's insulator function (3). Based on the finding that BORIS maps to the 20q13.2 region frequently altered in many malignancies, various cancer cell lines were screened for BORIS expression. BORIS transcripts were found in substantial proportions of a wide variety of tumor cell types (4). Using an extremely sensitive 2 step multiplex RT-PCR method, A. Lindblom et al., (Karolinska Hospital, Sweden) reported frequencies of BORIS expression of 80 and 88%, respectively, in 17 breast tumor cell lines and of 148 randomly selected primary breast cancer samples (A. Lindblom, unpublished). In contrast, the same team was unable to detect BORIS mRNA in any normal mammary tissues, or other normal somatic tissues. Based on these findings, and results indicating that BORIS protein can compete for CTCF binding sites in the *H19* imprinting control region, the globin FII insulator, and the c-*MYC* promoter, **we hypothesized that aberrant expression of BORIS may interfere with certain CTCF functions, thereby promoting cancer progression.**

To define the possible consequences of aberrant BORIS expression that may promote cancer progression in the human breast, we have been using a well-characterized human mammary epithelial cell (HMEC) culture model (reviewed in (5)). HMEC cultured from normal breast tissue display a finite life span, low or undetectable telomerase activity, and decreasing telomere length with passage (6). HMEC can spontaneously overcome a first RB-mediated, non-telomere length dependent proliferative arrest (stasis), associated with down-regulation of p16 expression (7). The resultant p53(+), p16(-) post-selection HMEC cease net proliferation when their mean terminal restriction fragment (TRF) length is ~5 kb. As cells approach this second proliferative barrier, telomere dysfunction is evidenced by the presence of widespread chromosomal aberrations, particularly telomeric fusions, and mitotic failures (8). In the p53(+) cultures, most cells remain viably arrested at all phases of the cell cycle, a growth arrest termed agonescence (8). When p53 is inactivated, populations display the massive cell death typical of crisis (J. Garbe et al., unpublished). Rare p53(+) and p53(-) immortal HMEC lines have been obtained following exposure to chemical carcinogens, over-expression of *c-myc* or *ZNF217* oncogenes, and/or a dominant negative p53 genetic suppressor element, GSE22 (9-11). Surprisingly, the newly immortal p53(+) lines initially show very low or undetectable telomerase activity and continue to divide with increasingly shortened mean TRF lengths. When the mean TRF length gets extremely short (<3 kb), growth becomes slow and heterogeneous. An extended process, termed conversion, ensues, during which telomerase activity and growth capacity gradually increase (12). In contrast, newly immortal p53(-) lines quickly display telomerase activity (11). Our studies indicate that overcoming telomerase repression and telomere dysfunction are rate-limiting factors in the malignant transformation of cultured HMEC. Once these barriers have been overcome, the resulting immortal lines can be induced to become growth factor- and anchorage-independent, as well as tumorigenic, by introduction of a variety of well-characterized oncogenes.

BODY

Task 1: Perform correlative studies of endogenous BORIS expression and DNA binding activity in HMEC at specific stages of immortal transformation. Our preliminary qualitative analysis of BORIS expression in HMEC, performed in collaboration with Dr. Victor Lobanenkov's lab (NIAID, NIH), indicated that this gene was not expressed in finite lifespan cultures derived from normal tissues, but that it was expressed in some cultures immortalized after exposure to a known chemical carcinogen or to specific oncogenes (data not shown). These data suggested that activation of BORIS expression might have been a cause or a consequence of changes accompanying immortalization. More recently, we have developed a quantitative RT-PCR assay in our own lab for more precise measurements of BORIS mRNA levels. We have used this quantitative assay to measure BORIS mRNA levels in human breast tumor cell lines and primary tumors. Levels of BORIS mRNA

in these samples were normalized to those of a gene transcript (TBP) commonly expressed at intermediate levels in most cells and tissues. While BORIS transcripts were easily detectable in normal testis by this method, they were very rare or undetectable in all breast tumor cell lines and primary tumor tissues examined, including those with elevated chromosome 20q13.2 copy numbers (**Fig. 1**). These assays were repeated with two independent PCR primer sets amplifying distinct regions of the BORIS transcript. The quantitative results indicated that the levels of BORIS gene expression in most human breast cell lines and primary tumors (< 1 transcript/cell) were unlikely to be sufficient to generate enough protein to interfere with CTCF function. The possibility remained, however, that there was discordance between BORIS mRNA and protein levels, and that BORIS transcripts at very low abundance were capable of generating significant amounts of protein with long half-life. To date, efforts to generate specific anti-BORIS antibodies have been unsuccessful, with one exception (2) that is limited in quantity and unavailable to us. A commercially available anti-BORIS antibody (Abcam) has recently become available. We generated a recombinant adenovirus encoding an HA-tagged BORIS protein, and used MCF7 cells infected with this virus to test the commercial antibody. The antibody did detect a band of the predicted size (83 kD) on an immunoblot of BORIS-transduced cells, but not in untransduced MCF7 or MDA-MB-231 cells (data not shown). These protein results confirmed our qRT-PCR results and strengthened our conclusion that, despite the presence of the BORIS gene in the amplified region of 20q, endogenous BORIS was unlikely to be expressed at sufficient levels to interfere with CTCF functions in most breast cancer cells.

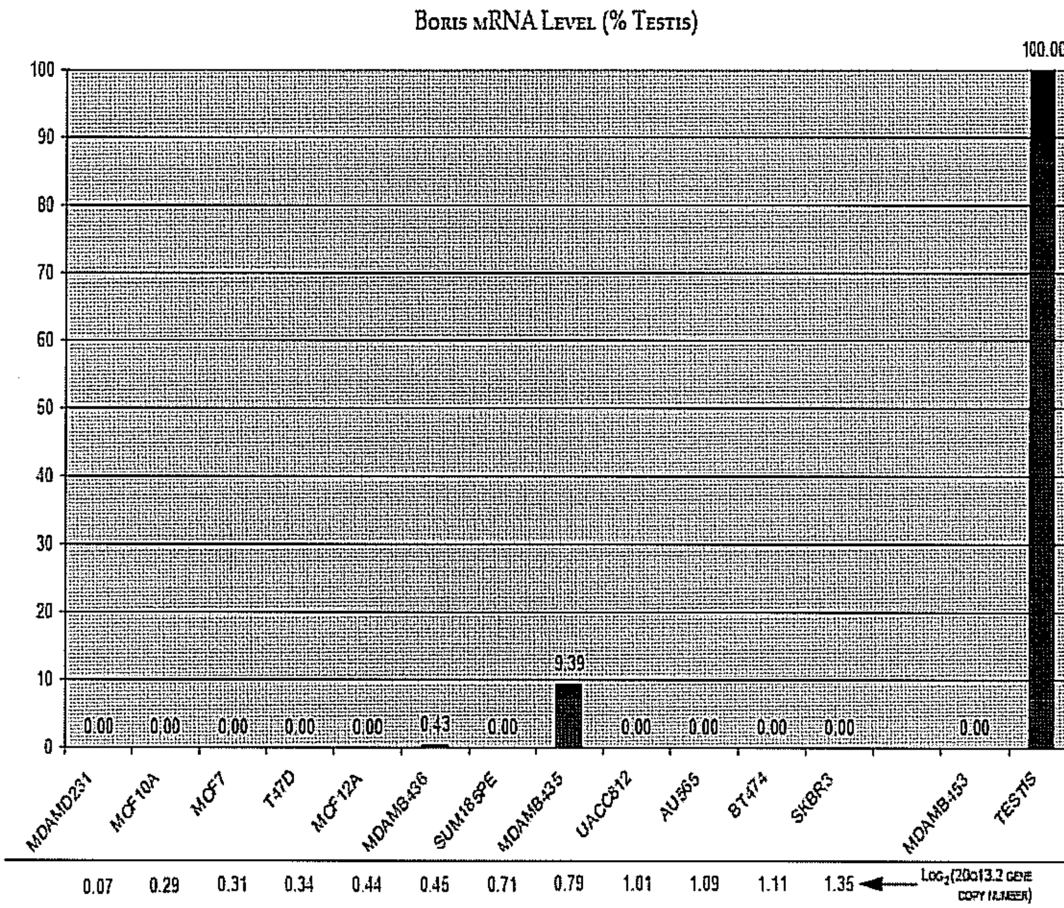


Figure 1. Expression of BORIS mRNA in human breast cancer cell lines. A quantitative RT-PCR assay was used to assay the levels of BORIS transcripts in the indicated cell lines. Raw Ct values were normalized to those of a constitutively expressed transcript (TBP) and compared to the normalized level in human testes. 20q13.2 gene copy numbers for the corresponding cell lines are presented below.

Task 2: Determine whether exogenous BORIS expression influences properties associated with conversion of p53(+) immortal HMEC. To address this problem, we introduced retroviruses containing BORIS cDNA or a control eGFP reporter gene into growing cultures of conditionally immortal 184A1 HMEC prior to conversion. Cell cultures were treated with selection agent G418. No significant differences in proliferation rate were noted between cells expressing the exogenously introduced BORIS gene and cells expressing the exogenously introduced eGFP reporter gene alone.

Task 3: Determine whether exogenous BORIS expression extends the proliferative potential of, or immortalizes finite lifespan HMEC when expressed alone or in combination with oncogenes *c-MYC* or *ZNF217*, or a dominant negative p53 genetic suppressor element. We tested the oncogenic properties of BORIS in HMEC directly by transducing the gene alone or in combination with additional changes that may act cooperatively with it. We transduced growing cultures of post-selection 184 HMEC with BORIS or a control vector. To monitor BORIS expression, we used a vector that contains an eGFP reporter gene linked to the

BORIS gene by an internal ribosome entry site (IRES). The IRES sequence allows the translation of two proteins from a single transcript. Since BORIS is located 5' to the eGFP coding region, all cells expressing eGFP green fluorescence are expected to also express high levels of BORIS protein. In each experiment, 3 plates of each condition were infected and monitored independently to control for jackpot effects and overgrowth by rare variants within a population. As shown in **Fig.2**, all three cell populations infected with the BORIS virus expressed very high levels of the transcript. In contrast, cells infected with the control vector did not show any expression of BORIS.

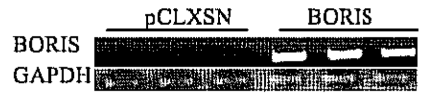


Figure 2. Expression of BORIS mRNA in 184 HMEC transduced with control vector (pCLXSN) or BORIS as determined by semi-quantitative RT-PCR. Expression of the housekeeping gene, GAPDH, was assayed as a control.

The transduced cells were monitored for population doubling times between subcultures, and morphological signs of agonescence or crisis. We did not observe any significant differences in cell morphology **(Fig.3)** or in growth rate **(Fig. 4)** initially after the infections. None of the cells transduced with BORIS showed detectable levels of hTERT prior to agonescence **(Fig. 5)**. However, one (SEBORIS2) of the three cell cultures infected with the BORIS virus yielded a single clone that overcame agonescence and became immortal. Cells originating from this clone retained functional p53 and gradually reactivated hTERT expression **(Fig. 5)**. We performed similar experiments in parallel, using finite lifespan pre-stasis HMEC and human fibroblasts derived from the same specimen. None of the cells transduced with BORIS immortalized in these latter experiments.

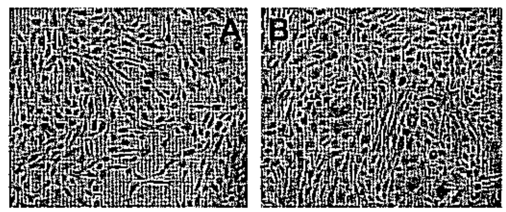


Figure 3. Morphology of post-selection 184 HMEC infected with (A) control, or (B) BORIS virus.

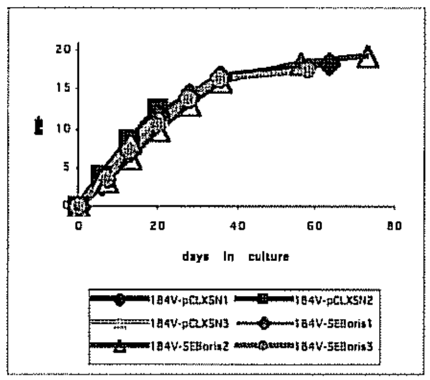


Figure 4. Growth rates of post-selection 184 HMEC infected with control (blue) or BORIS (red) containing viruses at passage 8 and grown until most cells stopped dividing.

Additional gene transfer experiments were performed using a second retroviral construct in which the BORIS gene was expressed as a fusion protein with eGFP at its C-terminus to directly track protein expression in real time. Cells transduced with this construct exhibited green fluorescence in the nuclei, as expected, whereas cells transduced with the control virus (containing the eGFP gene alone) showed fluorescent signal predominantly in the cytoplasms. Despite the high levels of BORIS-eGFP expressed by the cells in this experiment, no immortalization was observed.

Altogether, the above results suggest that BORIS alone is not an efficient immortalizing factor, but that under certain conditions it can cooperate with other unknown changes to immortalize normal finite lifespan cells. Aberrant BORIS expression may cooperate with other defects to enable cells to overcome agonescence and express telomerase. As one test of this hypothesis, we transduced growing cultures of post-selection HMEC with BORIS and/or the dominant negative p53 genetic suppressor element, GSE22. In this experiment, 3 plates of each condition were infected and monitored independently to control for jackpot effects and overgrowth by rare variants within a population. During the experiment, cells were monitored for morphological changes and population doubling times between subcultures, as well as signs of agonescence or crisis. In this experiment, we did not observe that cells infected with both BORIS and GSE22 had any advantage over the other cells in terms of growth rate. However, these cells demonstrated significant aberrations in cytokinesis, which were not detected in cells transduced with GSE22 alone **(Fig.6)**. p53 is known to play a role in control of cell division check points, as well as in processes controlling centrosome maturation and amplification. It is possible that lack of functional p53 in concert with BORIS over-expression can affect centrosome duplication during the cell cycle, leading to hyper-amplification. Centrosome amplification (the presence of more than two centrosomes at mitosis) is characteristic of many human cancers. Extra centrosomes

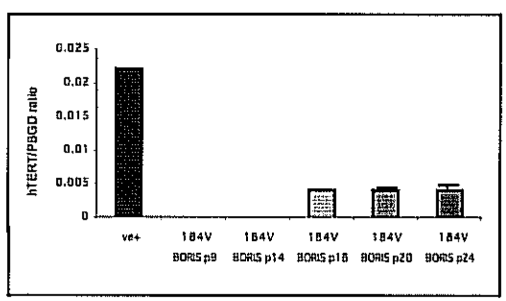


Figure 5. The levels of hTERT transcripts in 184 cells transduced with BORIS were analyzed by quantitative RT-PCR. The results were normalized to the expression of porfobilinogen deaminase – a constitutively expressed "housekeeping" gene.

can cause the assembly of multipolar spindles, which unequally distribute chromosomes to daughter cells resulting in genetic imbalances. The cells of most late-stage human cancers are aneuploid, genomically unstable and show high incidence of centrosome amplification. Genomic instability is thought to be a major driving force in multiple-step carcinogenesis. We hypothesize that in cells where it is expressed at sufficient levels, BORIS can be one of the proteins involved in control of centrosome duplication during the cell cycle. In accordance with this hypothesis, we have found that, in addition to their nuclear localization, BORIS-eGFP fusion proteins sometimes co-localize with δ-tubulin, a specific marker of centrosomes **(Fig.7A)**. HMEC cultures transfected with BORIS displayed higher percentages of cells with > 2 centrosomes than cultures transfected with control or GSE22 vectors alone, and these differences persisted with passage **(Figs.7B & 8)**.

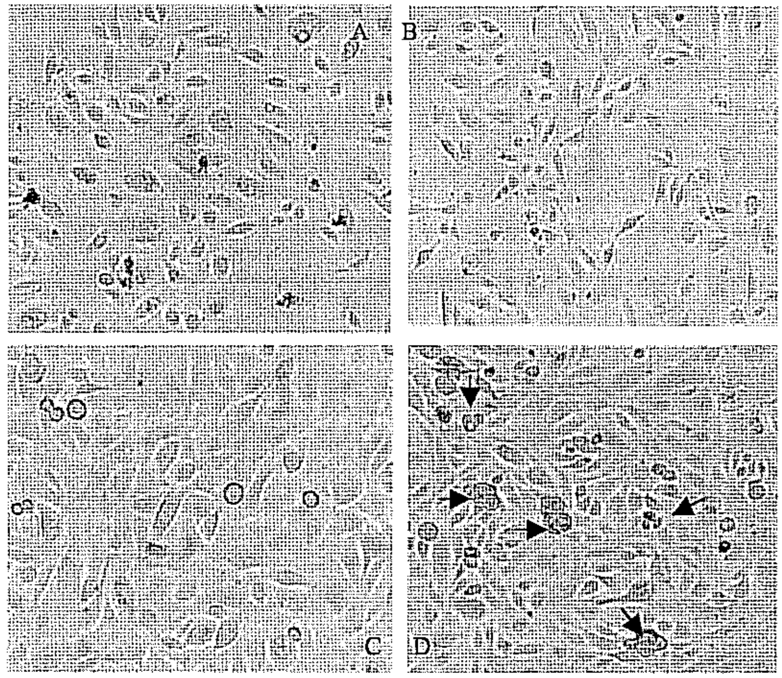


Figure 6. Morphology of 184 cells infected with: (A) BORIS/pBABE control; (B) pCLXSN control/pBABE control; (C) pCLXSN control/GSE22 (D) BORIS/GSE22. The arrows in panel D point to cells undergoing aberrant cytokinesis.

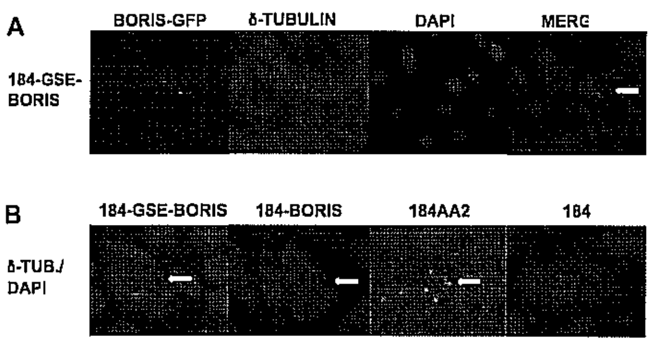


Fig. 7 A) Co-localization of BORIS-GFP and δ-tubulin in 184-GSE22 cells transfected with BORIS-GFP; B) Cells displaying abnormal numbers of δ-tubulin positive centrosomes in cultures transfected with GSE22 and BORIS (184-GSE-BORIS), transfected with BORIS alone (184-BORIS), or immortalized after p53 inactivation (184AA2). A representative cell displaying 2 centrosomes in a control culture (184) is shown for comparison. Arrows indicate cells with ≥3 centrosomes.

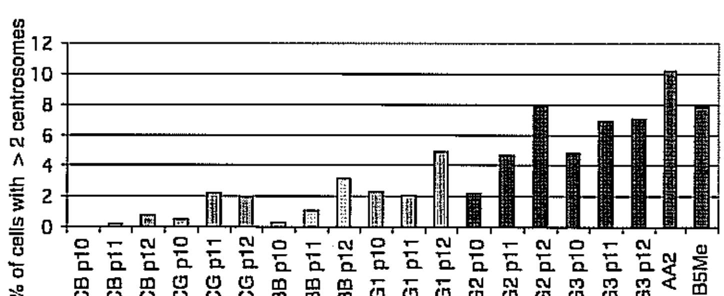


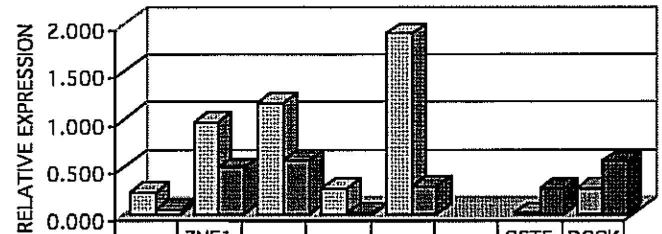
Figure 8. Quantitation of percentages of cells with > 2 centrosomes in 184 cells infected with: pCLXSN control/pBABE control (CB); pCLXSN control/GSE22 (CG); BORIS/pBABE (BB); BORIS/GSE22 (BG) retroviruses and assayed at passages (p) 10-12. Immortally transformed HMEC lines AA2 and B5Me were assayed for comparison.

Task 4: Determine whether exogenous BORIS expression influences additional phenotypic properties of normal or immortalized HMEC.

A) Anchorage dependence: Anchorage-independent growth (AIG) is often exhibited by cells derived from human breast tumors or by cells that have been tumorigenically transformed *in vitro*. To determine if BORIS is able to promote AIG we suspended retrovirally transduced immortal HMEC in 1.5% methylcellulose in growth medium. The BORIS expression level in cells used in the experiment was determined by semi-quantitative RT-PCR prior to plating. The cells were fed weekly and, at 4 weeks post-plating, the plates were visually inspected for the presence of cell colonies displaying AIG. No such colonies were detected in cultures of immortal HMEC

over-expressing BORIS (data not shown), suggesting that BORIS alone is not sufficient to confer this phenotype.

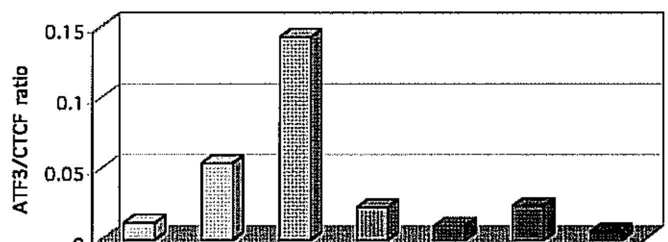
B) Gene expression: To determine whether BORIS directly or indirectly influences cellular transcription, we have performed expression microarray experiments using a new state-of-the-art Affymetrix HTA GeneChip system recently purchased by LBNL's Life Sciences Division. This high throughput facility presently employs U133A 2.0 chips of reduced feature size in a 96-well format. We transduced 184 HMEC with either BORIS or control retroviruses and harvested RNA after a brief selection. Microarray analysis was performed using duplicate samples, and the resulting data were analyzed using commercial GeneTraffic software (Stratagene). Genes which showed consistent differences of > 2 fold compared with controls were selected for further study. PCR primers were designed for several of these genes, and altered regulation was confirmed by semi-quantitative RT-PCR **(Fig.9)**. Transient transfections were performed in 184 HMEC with increasing amounts of the BORIS-IRES-eGFP or control plasmids, and the harvested RNA subjected to quantitative RT-PCR to further document the dependence of selected gene transcripts on BORIS expression. The example shown in **Fig.10** shows the direct correspondence between amount of BORIS plasmid transfected and ATF3 transcripts expressed. ATF3 is of particular interest because it is a member of the CREB protein family of transcription factors. In addition to ATF3, a number of early growth response genes, including the oncogenes Fos and Jun, appear to be significantly upregulated by BORIS expression. Also of interest is the up-regulation of ZNF165, which like BORIS itself, has been reported to be a cancer-testis antigen.



GENE	EGR2	ZNF1 65	ID2	ATF3	FOSB		GSTE 1	DOCK 4
BORIS	0.236	0.972	1.17	0.27	1.9		0.02	0.28
pCLXSN	0.046	0.484	0.56	0.01	0.29		0.28	0.56

Figure 9. Semi-quantitative RT-PCR results for genes that showed differential expression in 184 HMEC transduced with BORIS vs. control retroviruses.

ATF3 level in 184V transiently transfected with BORIS



	.5 BORIS	1 BORIS	2 BORIS	.5 IRES_EGFP	1 IRES_EGFP	2 IRES_EGFP	untransfected
Series1	0.012	0.054	0.143	0.023	0.01	0.024	0.006

Figure 10. Transient transfections with increasing concentrations of BORIS-IRES-eGFP plasmid (0.5, 1, and 2 μg/60 mm dish) and empty vector at the same concentrations confirm that BORIS regulates ATF3 expression. The cells were harvested 24 hrs. after transfection with the indicated plasmids, and the levels of ATF3 mRNA were quantified by quantitative RT-PCR. The levels were then normalized using the levels of CTCF transcripts for normalization.

Task 5: Determine whether interference with endogenous BORIS expression blocks growth and/or telomerase expression in immortalized HMEC. BORIS expression may or may not be continuously required for maintenance of an altered phenotype in transformed cells. To address this problem we transduced immortal HMEC expressing high levels of endogenous BORIS with retroviral constructs encoding gene-specific short hairpin RNAs (shRNAs) to suppress BORIS expression. To date, it has not proved possible to predict which gene-specific sequences work best as shRNAs, so we used constructs containing sequences from different regions of the BORIS gene: siBORIS772 and siBORIS1594. After antibiotic selection, BORIS mRNA levels in shRNA-retrovirus infected cell lines were determined by semi-quantitative RT-PCR **(Fig. 11)**. Cells transduced with siBORIS772 virus showed about 50% reduction of BORIS level as compared to parental cells, and are currently being used in ongoing experiments.

KEY RESEARCH ACCOMPLISHMENTS

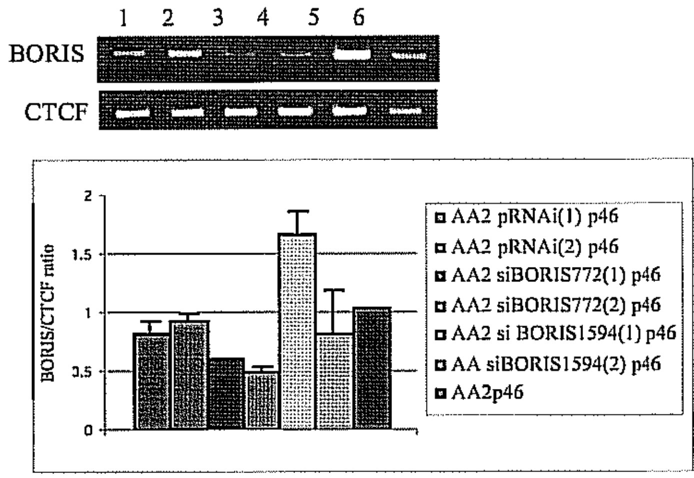


Figure 11. BORIS mRNA expression in 184AA2 HMEC was quantitated after transduction with BORIS-specific shRNA retroviruses. The semi-quantitative RT-PCR results were normalized to the levels of CTCF transcripts in the same cells. pRNAi is a non-specific control.

• We have developed a quantitative RT-PCR assay for BORIS mRNA expression, and have determined that the levels of BORIS expressed in most human breast cancer cell lines and tumors are unlikely to be sufficient to compete with CTCF for binding to CTCF sites.

• Our results suggest that BORIS alone is not an efficient immortalizing factor, but that in rare instances it might cooperate with additional factors to induce immortalization.

• Cells infected with both BORIS and GSE22 (a dominant negative p53 genetic suppressor element) demonstrated significant aberrations in cytokinesis, which were not detected in cells transduced with GSE22 alone.

• In addition to their nuclear localization, BORIS-eGFP fusion proteins sometimes co-localized with δ-tubulin, a specific marker of centrosomes.

• HMEC cultures transfected with BORIS displayed higher percentages of cells with > 2 centrosomes than cultures transfected with control or GSE22 vectors alone, and these differences persisted with passage.

• Microarray and RT-PCR analyses have identified several key early growth response genes as well as a cancer-testis antigen gene whose regulation was altered by BORIS expression in HMEC.

REPORTABLE OUTCOMES

None to date

CONCLUSIONS

Our results to date indicate that despite its presence in the frequently amplified region of chromosome 20q, the BORIS gene is rarely expressed at significant levels in most human breast cancers, and therefore is unlikely to play a significant etiologic role in this disease. However, our results do indicate that in the rare cases where BORIS is aberrantly expressed, it may cooperate with other changes (e.g. p53 inactivation) to destabilize cellular genomes. Since BORIS-eGFP protein sometimes co-localizes with centrosomes, it is possible that BORIS expression can cause genomic instability through aberrant affects on centrosome duplication during the cell cycle. BORIS expression may also cause genomic instability through its significant effects on the regulation of several key early growth response genes.

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